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(54) Title: AN IMMUNOINTERACTIVE MOLECULE WHICH BINDS THE *TIE2*/TEK RECEPTOR EXTRACELLULAR DOMAIN

(57) Abstract

The present invention relates to molecules which are immunointeractive with an animal growth factor receptor and, more particularly, to animal *tie2*/Tek receptor. The immunointeractive molecules provide the basis for new therapeutic and diagnostic agents which are useful in the treatment, prophylaxis and diagnosis of angiogenic-dependent phenotype.

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AN IMMUNOINTERACTIVE MOLECULE WHICH BINDS THE TIE2/TEK RECEPTOR EXTRACELLULAR DOMAIN

FIELD OF THE INVENTION

The present invention relates generally to immunointeractive molecules to an animal growth factor receptor and, more particularly, to animal *tie*2/Tek receptor. The immunointeractive molecules provide the basis for a new range of therapeutic and diagnostic agents for use such as in the treatment, prophylaxis and diagnosis of an angiogenic-dependent phenotype.

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description. Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined following the bibliography.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

BACKGROUND TO THE INVENTION

Angiogenesis is the formation of new blood vessels from those which preexist within the body (1). It is of fundamental importance for the development of the embryo and a number of roles in post-natal life (e.g. wound healing, tissue regeneration, cyclical growth of the corpus luteum and endometrium). Angiogenesis is also important in a number of pathological conditions including the growth of solid tumours (1). Recent studies have suggested that the acquisition of an angiogenic-dependent phenotype is a key factor in the development of metastasis.

The introduction of techniques based on the polymerase chain reaction (PCR) for amplifying protein tyrosine kinase sequences has enabled the rapid isolation of novel members of the growth factor receptor family. Two of the putative receptors isolated NYK/flk-1 receptor (2) [neuroepithelial kinase/fetal liver kinase] and *tie*2/Tek (3) have been shown to be expressed on endothelial cells and their precursors. NYK and its human equivalent KDR (4) have been shown to bind and be activated by the endothelial cell mitogen VEGF/VPF (vascular endothelial growth factor/vascular

permeability factor) (5). VEGF has a mitagenic effect on endothelial cells but is also a potent mediator of vascular permeability. VEGF has in recent studies been shown to play a role in the hypoxia induced angiogenesis seen in a glioma model suggesting that VEGF may play a role in mediating angiogenesis in other tumour systems (6). Other studies have shown that anti-VEGF monoclonal antibodies have an anti-tumour effect *in vivo* (7).

In work carried out since the original isolation of the *tie*2 cDNA, other groups havfe shown *tie*2 to be expressed largely in cell lines of endothelial origin and in the development of the mouse embryo (8) in haematopoietic cells (9), and in mouse embryonic stem cells which are undergoing differentiation (10). In embryos in which the action of the receptor was abated by either expression of a dominant negative or a null allele, vasculogenesis is severely impaired, resulting in a reduction in the number of endothelial cells at days 8.5 and 9.0 (11).

In work leading up to the present inventions the inventors developed a series of antibodies to the NYK (VEGFR2) receptor extracellular domain. These antibodies are useful in the development of a new range of therapeutic molecules such as agonists and antagonists to NYK-receptor interaction as well as a range of diagnostic agents capable of, for example, detecting normal, abnormal or mutant receptors, receptor expression on a cell surface and/or receptor-ligand interaction.

SUMMARY OF THE INVENTION

One aspect of the present invention contemplates an immunointeractive molecule capable of binding or otherwise associating with an animal *tie*2/Tek receptor extracellular domain. Preferably, the animal is a mammal such as a human or murine species. Preferably, the immunointeractive molecule is a polyclonal or monoclonal antibody.

Another aspect of the present invention is directed to a diagnostic agent comprising an immunointeractive molecule capable of binding or otherwise associating with an animal *tie*2/Tek receptor immunointeractive molecule is an antibody labelled with a reporter molecule.

Yet another aspect of the present invention relates to a pharmaceutical composition comprising an immunointeractive molecule as contemplated above together with one or more pharmaceutically acceptable carriers and/or diluents.

Still yet another aspect of the present invention provides a method for treating an angiogenic-dependent phenotype or disease condition resulting therefrom in a mammal, said method comprising administering to said mammal an effective amount of an immunointeractive molecule capable of binding or otherwise associating with an animal *tie*2/Tek receptor is particularly useful in the treatment of metastasis.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a photographic representation of a silver stained 7.5% SDS PAGE gel showing the analysis of purified *tie*2-FLAG™ construct eluted from the M2 affinity column. The elution of the bound protein is shown with the concentration of the peptide solution added shown at the top of the gel.

Figure 2 is a photographic representation demonstrating detection of 0.5 μg of *tie*2-FLAG™ protein by Western blotting analysis using the monoclonal antibodies raised against *tie*2-FLAG. The positive control antibody (M2) is directed to the FLAG™ epitope. The negative control antibody was directed to the extracellular domain of the receptor tyrosine kinase NYK. The molecular weight standards are represented in kilodaltons.

Figure 3 is a graphical representation of an analysis of shared epitopes of the anti-*tie*2 monoclonal antibodies by the use of real time interaction on a biosensor. RU represents Response Units, which are proportional to the concentration of protein at the surface of the chip. The baseline level relates to the amount of *tie*2-FLAG™ bound to the chip at t=0 seconds (s). Addition of the antibody 3a6 at time 250s and 6a12 at 950s both result in increased RU levels, indicating binding of the two mAbs. However, the addition of 1e11 antibody does not result in increased RU after the cessation of sample flow at 2450s. The addition of antibody 4g8 results in a large increase of RU values at 2900s implying significant antibody binding. Other combinations of anti-*tie*2 antibodies have shown that the epitopes of 6a12 and 1e11 are shared. Control experiments confirmed that saturating amounts of the antibodies were used in these experiments.

Figure 4 is a graphical representation showing analysis of the binding of the anti-*tie*2-FLAG™ binding to native (a, b and c) and denatured (d, e and f) *tie*2-FLAG™ on the biosensor. The response of the chip is shown in the figure with each graph representing the response in RU on the Y-axis versus time (X-axis). An increase in RU corresponds to antibody binding to the *tie*2-FLAG™ derivatised chip. Purified antibody was diluted in biosensor running buffer at saturating concentrations and added at t=120s. Biosensor running buffer itself causes no change in refractive index when the sample is being applied. The dip in all traces at t=720s is the desorption of the bound material using 50 mM diethylamine pH 12. *tie*2-FLAG™ was denatured by passing a solution of 1.5% v/v 2-mercaptoethanol dissolved in 6 M guanidine-HCl and 1 M Tris-HCl pH 8.9. Antibody 1e11 demonstrates no binding to the denatured *tie*2-FLAG™ immobilised on the biosensor chip (Figure 4d).

Figure 5 is a graphical representation of response of the *tie*2-EpoR expressing Ba/F3 cell line and a control Ba/F3 cell line to the 1e11 anti-*tie*2 antibody. 10^4 cells were incubated in 100 μl of growth medium lacking IL-3 with various concentrations of antibody, allowed to incubate for 4 days, then the number of proliferating cells ascertained by the use of a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (12). The result of the MTT assay is expressed in the difference in absorbances at 690 nm and 560 nm, which is proportional to the number of proliferating cells in the sample. The control cells were not affected by the antibody, whereas the *tie*2-EpoR expressing cells are stimulated to proliferate.

Figure 6 is a graphical representation showing detection of the *tie*2 receptor expressed on Ba/F3 cells using the 4g8 antibody and FACS analysis. The X-axis represents channel number and relates to the amount of fluorescence detected, while the Y axis shows the number of cells corresponding to that level of fluorescence. The unshaded profile represents the fluorescence of untransfected Ba/F3 cells and the shaded region that of cells transfected with the *tie*2 construct. Binding of the 4g8 antibody was detected using a FITC-labelled anti-rat Ig antibody. Cells were analysed using a Becton Dickinson FACScan unit.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the present invention provides an immunointeractive molecule capable of binding or otherwise associating with an animal *tie*2/Tek receptor, and in particular, the extracellular domain of such a receptor.

Preferably, the immunointeractive molecules are in the form of antibodies such as polyclonal or monoclonal antibodies although monoclonal antibodies are preferred. The present invention also extends to immunologically interactive fragments, parts, derivatives, homologues or analogues of these antibodies. Such antibodies may be in isolated or purified form meaning that a composition comprises at least 25%, more preferably at least 35%, even more preferably at least 45-50%, still more preferably at least 60-75% and even still more preferably at least 95-100% of the antibodies as determined by weight, immunoreactivity or other convenient means. Alternatively, the antibodies may be present in the form of isolated culture supernatant, tissue extract, serum, whole blood or ascites fluid.

Preferably, the animal *tie*2/Tek receptor is of mammalian origin such as from a human, livestock animal (e.g. cow, horse, sheep, goat or donkey), laboratory test animal (e.g. rat, mouse or rabbit), companion animal (e.g. dog or cat) or captive wild animal (e.g. dingo, fox, wild boar or kangaroo). The most preferred receptors are of human and laboratory test animal origin (e.g. mouse).

Where the antibodies are polyclonal antibodies, they may be generated in any convenient host including a human, livestock animal, companion animal or captive wild animal as hereinbefore described. Where the antibodies are monoclonal antibodies, they may be prepared in any convenient hybridoma such as of murine (e.g. rat or mouse) origin.

The receptor used to generate the antibodies may be the whole receptor such as in purified, partially purified or isolated form including in the form of isolated membrane preparations. The receptor may also be produced by recombinant procedures or synthetic procedures or a combination thereof. In a particularly preferred embodiment, a fragment of the receptor is used which, in an even more preferred embodiment, is fused to a suitable carrier or marker molecule such as FLAG™ protein or alkaline phosphatase CAP. Another carrier is glutathione-S-transferase (GST).

According to this preferred embodiment, there is provided a molecule interactive with a non-full length *tie*2/Tek receptor fused to a carrier molecule. The non-full length portion of the receptor acts an immunoreactive molecule. Preferably, the non-full length receptor comprises its extracellular domain. Preferably, the carrier molecule is FLAG™ or AP.

The resulting fusion molecule is then used to generate polyclonal or monoclonal antibodies which may undergo immunoadsorbent procedures to provide a composition of substantially, for example, extracellular domain-reactive receptor antibodies.

The term "immunointeractive molecules" is used herein in its broadest sense and includes antibodies, parts, fragments, derivatives, homologues or analogues thereof, peptide or non-peptide equivalents thereof and fusion molecules between two or more antibodies or between an antibody and another molecule. The antibodies or other immunointeractive molecules may also be in recombinant or synthetic form.

Accordingly, the present invention contemplates mutants and derivatives of the immunointeractive molecules, especially when such molecules are antibodies. Mutants and derivatives of such antibodies include amino acid substitutions, deletions and/or additions. Furthermore, amino acids may be replaced by other amino acids having like properties, such as hydrophobicity, hydrophilicity, electronegativity, bulky side chains, interactive and/or functional groups and so on. Glycosylation variants and hybrid antibodies are also contemplated by the present invention.

Amino acid substitutions are typically of single residues; insertions will usually be of the order of about 1-10 amino acid residues; and deletions will range from about 1-20 residues. Deletions or insertions preferably are made in adjacent pairs, i.e: a deletion of 2 residues or insertion of 2 residues.

The amino acid variants referred to above may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. Techniques for making substitution mutations at predetermined sites in DNA having known sequence are well known, for example through M13 mutagenesis. The manipulation of DNA sequences to produce variant proteins which manifest as substitutional, insertional or deletional variants are well known in the art.

Other examples of recombinant or synthetic mutants and derivatives of the antibodies of the present invention include single or multiple substitutions, deletions and/or additions to any molecule associated with the ligand such as carbohydrates, lipids and/or proteins or polypeptides. Naturally occurring or altered glycosylated forms of the subject antibodies are particularly contemplated by the present invention.

Amino acid alterations to the subject polypeptide contemplated herein include insertions such as amino acid and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino or carboxyl terminal fusions, of the order of about 1 to 4 residues. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Such substitutions may be made in accordance with Table 1.

TABLE 1

Original Residue	Exemplary Substitutions
Ala	Ser
Arg	Lys
Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Val
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile
Pro	Ser; Ala
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

The terms "analogues" and "derivatives" also extend to any functional chemical equivalents of the antibodies characterised by their increased stability and/or efficacy *in vivo* or *in vitro*. The terms "analogue" and "derivatives" further extend to any amino acid derivative of the antibodies as described above.

Antibody analogues contemplated herein include, but are not limited to, modifications to side chains, incorporation of unnatural amino acids and/or

derivatising the molecules and the use of crosslinkers and other methods which impose conformational constraints on the antibodies. Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5'-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3- butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbomoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during protein synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-

butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids.

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n = 1 to n = 6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides could be conformationally constrained by, for example, incorporation of C_α and N_α-methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

The present invention, therefore, extends to amino acid and/or chemical analogues of the subject antibodies having the identifying characteristics of being interactive with the extracellular domain of the *tie*2/Tek receptor.

Accordingly, reference herein to an antibody includes the naturally occurring molecule, recombinant, synthetic and analogue forms thereof and to any mutants, derivatives and human and non-human homologues thereof including amino acid and glycosylation variants.

The immunointeractive molecules of the present invention may be used to develop a new range of therapeutic and diagnostic agents. For example, the antibodies or fragments or derivatives thereof may act as antagonists and be useful, for example, in the treatment of angiogenic-dependent phenotype and disease conditions resulting therefrom (e.g. metastasis). They may also be used for screening for agonists useful, for example, where angiogenesis is to be promoted. Normal, abnormal or mutant receptor structure or receptor expression can also be determined through immunoreactivity studies.

According to this latter embodiment, there is contemplated a method of detecting a *tie*2/Tek receptor on a cell in a biological sample, said method comprising contacting said sample with an immunointeractive molecule capable of binding to the extracellular domain of said *tie*2/Tek receptor immobilised to a solid support for a

time and under conditions sufficient for an immunointeractive molecule in *tie*2/Tek complex to form and then detecting the presence of said complex.

In one preferred method, the immunointeractive molecule in *tie*2/Tek complex is detected by contacting the complex with an antibody against the immunointeractive molecule with the antibody being labelled with a reporter molecule. Alternatively, the immunointeractive molecule itself is labelled with a reporter molecule.

The immunointeractive molecules are generally antibodies and these or antibodies directed against the immunointeractive molecules may be polyclonal or monoclonal antibodies and both are obtainable by immunisation of a suitable animal with the immunointeractive molecule (or *tie*2/Tek molecule) and either type is utilisable in the assay. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of immunointeractive molecule or *tie*2/Tek preparation, or antigenic parts thereof, collecting serum from the animal, and isolating specific antibodies by any of the known immunoadsorbent techniques. Although antibodies produced by this method are utilisable in virtually any type of assay, they are generally less favoured because of the potential heterogeneity of the product.

The use of monoclonal antibodies in the above immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitised against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art. (See, for example Douillard and Hoffman, Basic Facts about Hybridomas, in *Compendium of Immunology* Vol II, ed. by Schwartz, 1981; Kohler and Milstein, *Nature 256:* 495-499, 1975; *European Journal of Immunology 6:* 511-519, 1976).

The presence of a *tie*2/Tek receptor may be accomplished in a number of ways such as by Western blotting and ELISA procedures. A wide range of immunoassay techniques are available as can be seen by reference to U.S. Patent Nos. 4,016,043, 4, 424,279 and 4,018,653. These, of course, include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the traditional

competitive binding assays. These assays also include direct binding of a labelled antibody to a target.

Sandwich assays are among the most useful and commonly used assays and are particularly useful in the present invention. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an immunointeractive molecule is brought into contact with a biological sample comprising cells potentially carrying *tie*2/Tek. After a suitable period of incubation, for a period of time sufficient to allow formation of an immunointeractive molecule - *tie*2/Tek complex, an antibody specific to the immunointeractive molecule, labelled with a reporter molecule capable of producing a detectable signal, is then added and incubated allowing sufficient time for the formation of a tertiary complex. Any unreacted material is washed away, and the presence of the antibody bound to the immunointeractive molecule is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of hapten. Variations on the forward assay include using an immunointeractive molecule labelled with a reporter molecule. In addition, the immunointeractive molecule or cells may be immobilised onto a solid support.

Suitable solid supports include glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing mLBP to the polymer.

"Reporter molecule", as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

In the case of an enzyme immunoassay, an enzyme is conjugated to the immunointeractive molecule or an antibody thereto generally by means of glutaraldehyde or periodate. As will be readily recognised, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable colour change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates which yield a fluorescent product rather than the chromogenic substrates noted above. Generally, the enzyme-labelled antibody is added to the immunointeractive molecule-receptor complex, allowed to bind, and then the excess reagent is washed away. Alternatively, an enzyme labelled immunointeractive molecule is used. A solution containing the appropriate substrate is then added to the tertiary complex. The substrate will react with the enzyme linked to the antibody/immunointeractive molecule, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. "Reporter molecule" also extends to use of cell agglutination or inhibition of agglutination such as red blood cells on latex beads, and the like.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. Immunofluorescence and EIA techniques are both very well established in the art and are particularly useful for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

By detecting *tie*2/Tek receptors by the procedures, aberrant receptors may be discerned thus providing a useful screening procedure for potential disease conditions.

The present invention also provides a pharmaceutical composition comprising an effective amount of an immunointeractive molecule capable of binding or otherwise associating with the extracellular domain of *tie*2/Tek receptor and one or more pharmaceutically acceptable carriers and/or diluents. The active ingredients of a pharmaceutical composition comprising the immunointeractive molecules are contemplated to exhibit excellent therapeutic activity, for example, in the treatment of angiogenic-dependent phenotype and disease conditions resulting therefrom, such as metastasis, in an amount which depends on the particular case. For example, from about 0.5 μg to about 20 mg per kilogram of body weight per day may be administered. Dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active compound may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, intradermal or suppository routes or implanting (e.g. using slow release molecules). Depending on the route of administration, the active ingredients which comprise the immunointeractive molecules may be required to be coated in a material to protect said ingredients from the action of enzymes, acids and other natural conditions which may inactivate said ingredients. In order to administer the immunointeractive molecules by other than parenteral administration, they will be coated by, or administered with, a material to prevent its inactivation. For example, the immunointeractive molecules may be administered in an adjuvant, co-administered with enzyme inhibitors or in liposomes. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes.

The active compounds may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and

use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When immunointeractive molecules are suitably protected as described above, the active, compound may be orally administered, for example, with an inert diluent

or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 10 μg and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the following: A binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

As used herein "pharmaceutically acceptable carrier and/or diluent" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use

thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired as herein disclosed in detail.

The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 0.5 μg to about 2000 mg. Expressed in proportions, the active compound is generally present in from about 0.5 μg to about 2000 mg/ml of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

In a most preferred embodiment, the immunointeractive molecules used in a pharmaceutical composition are antibodies or mutants or derivatives thereof. Most preferably, the antibodies are monoclonal antibodies.

The present invention is further described by reference to the following non-limiting examples.

EXAMPLE 1

Cell Culture

The murine myeloma P3X63Ag8.653(NS-1) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% v/v HI FBS, 5 mM L-glutamine, 50 μg/ml gentamycin, grown at 37°C in a humidified atmosphere of 10% v/v CO_2.

Hybridomas were grown in supplemented DMEM plus 10 μg/ml of recombinant IL-6 (rIL-6) at all stages after removal from the hypoxanthine aminupterin thymidine (HAT) selection medium. NIH-3T3 cells transfected with the AP-TAG- 1 constructs were selected in supplemented DMEM containing 700 μg/ml G418. CHO cell lines were grown in Glasgow Minimal Essential Medium supplemented with 50 μg/ml gentamycin, non-essential amino acids, sodium pyruvate, glutamate and asparagine, nucleosides and 25 mM methionine sulphoxide (MSX). COS cells were maintained in RPMI-1640 medium with supplements at 37°C and 5% v/v $C0_2$ and transfected using the DEAE-dextran protocol.

EXAMPLE 2

Construction of the *tie2*-FLAG™ Plasmid

The full length clone of the mouse *tie2* receptor described by Runting *et al.*, (3) was subcloned into the mammalian expression vector pCDM8 (Invitrogen, San Diego, CA) using the *BstXI* restriction enzyme site. Single stranded DNA was generated using the M13 origin of replication, and this DNA used as a template to make *tie2* cDNA containing specific enzyme sites. In frame *BamHI* sites were introduced prior to the transmembrane domain to allow ligation into the (5')*HindIII*-(3')*BglII* site of the expression vector AP-TAG- 1. The mutant *tie2* receptor containing the *BamHI* site located at the junction of the extracellular domain and the transmembrane domain (NYK-EX-*BglII*) was used to ligate a oligonucleotide linker sequence encoding the FLAG™ marker peptide, an in frame stop codon, *BglII* compatible ends and an internal *ClaI* site. (IBI; 5' GATCTGACTACAAGGACGACGATGACAAGTGAATCGATA [SEQ ID NO: 2]), ((N)Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-Term(C) [SEQ ID NO: 1]). The *tie2*-FLAG™ cDNA was then transferred (5' *XbaI*-3' *XbaI*) into the *XbaI* site of the CHO cell expression vector. This construct was transfected into CHO-K1 cells and positive clones selected in medium containing 25 mM methionine sulphoxide (MSX). Expressing clones were selected by immunoprecipitation of ^{35}S-methionine labelled cells with anti-FLAG™ (M2) antibody gel.

EXAMPLE 3

Affinity Chromatography

*tie*2-FLAG™ was purified from the expended tissue culture supernatant of *tie*2-FLAG™-CHO by affinity chromatography on a M2 (anti-FLAG) gel (IBI). Supernatant (100 ml) was passed over the M2 column then subsequently washed with 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02% Tween 20 v/v (optional); 50 mM TEA pH 10.0, 150 mM NaCl 0.02% Tween v/v 20 and again with 1.0 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02% Tween 20. Bound material was eluted with either 100 mM glycine-HCl pH 3.0 (neutralised in 1/10 volume 1 M Tris-HCl pH 8.6) or 25 μg/ml FLAG™ peptide (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C [SEQ ID NO: 1]) in 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02% v/v Tween 20 (Refer figure 1). Both of these elution techniques gave about 90-95% pure *tie*2-FLAG™ as determined by SDS-PAGE. For immunisations and coupling to the sensor chip these proteins were further purified by ion-exchange chromatography (MonoQ, HPLC 1090) to give a single homogeneous species corresponding to *tie*2-FLAG™.

EXAMPLE 4

Monoclonal Antibody Production

Purified *tie*2-FLAG™ (approximately 10 μg) was used to immunise female Wistar rats on day 54 (i.p.), 24 (i.p.) and 3 (i.v. and i.p.) prior to fusion with the mouse myeloma P3X63Ag8.653 (NS-1). *tie*2-FLAG™ was prepared for the first two i.p. immunisations by combining with adjuvant containing trehalose dimycolate from *Mycobacterium phlei,* monophosphoryl lipid A from *Salmonella minnesota* R595, PBS/0.2% v/v Tween 80 and squalene as described in the manufactures instructions (RIBI Immunochem Research, Hamilton, MT). The final immunisation was performed with purified *tie*2-FLAG™ diluted 1/1 with PBS. Rats were test bled on day 18 prior to fusion and the titre of anti-*tie*2-FLAG™ antibodies determined by a solid phase EIA and immunoprecipitation of *tie*2-AP.

Monoclonal antibodies to the *tie*2 extracellular domain were selected by screening the fusion on purified *tie*2-FLAG™ and NYK extracellular domain-FLAG™ by an enzyme immunoassay. Briefly, 96 well PVC microtitre plates were coated with either *tie*2-FLAG™ or NYK-EX-FLAG™ at a level previously determined by reactivity

with an anti-FLAG™ antibody (M2) to give an equivalent signal. Hybridoma supernatants were added and incubated for 2 h at 4°C, followed by six washes with PBS/0.02% v/v Tween 20 (buffer). Incubation with a horse radish peroxidase conjugate α anti-rat Ig followed for 1 h at 4°C. After washing, the assay was developed with an ABTS substrate system and the assay quantitated by reading absorbances at 405 mm in a multiwell plate reader (Flow Laboratories MCC/340, McLean, VA). Antibodies selected for further analysis were subcloned three times by limiting dilution. These antibodies were designated 3g1, 4g8, 6a12, 1e11 and 3a6.

EXAMPLE 5

Purification of Rat Monoclonal Antibodies

Rat monoclonal antibodies were purified using the technique of Darby *et al.* (13). Briefly, FCS was depleted of bovine IgG by serial passage over a protein G-Sepharose fast flow gel. The resulting bovine IgG depleted serum was used for the bulk culture of the rat hybridoma cell line. Cells were grown in roller bottles containing DMEM, 10% w/v IgG depleted FCS, 5 mM glutamine, 50 μg/ml gentamicin and 10 μg/ml recombinant IL-6 until expiration and the supernatant removed from the cellular debris. Rat IgG was subsequently purified by affinity chromatography on protein G-Sepharose and the yield assessed by OD_{280} nm.

Antibodies were dialysed versus PBS for use in assays. For coupling to CNBr-activated Sepharose beads (Pharmacia) the antibodies were dialysed against 0.1 M $NaHCO_3$/0.5 M NaCl and then conjugated at about 2 mg of antibody per ml of beads. Protein A purified rabbit Ig was also coupled to the Sepharose beads as a non-binding control.

EXAMPLE 6

Cell Labellings and Immunoprecipitations

Subconfluent monolayers of either NIH-3T3 fibroblasts or CHO-K1 cells transfected with the various *tie2* extracellular domain constructs were starved in Met^-Cys medium containing 5% v/v FCS, 5 mM L-glutamine and 50 μg/ml gentamycin for 0.5 h prior to incubating with 100 μCi ^{35}[S] cysteine/methionine (Translabel, ICN) per 10^6 cells. After a 16 h incubation at 37°C, supernatants were collected and used

for immunoprecipitation analysis. Supernatants were incubated with 10 μl of anti-FLAG™ M2 affinity gel with rotation for 24 hours. Immunoprecipitates were washed three times with 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% v/v Triton X-100 and then twice with 10 mM Tris HCl pH 8.0, 150 mM NaCl, 0.1% v/v Triton X-100 then 10 mM Tris-HCl pH 8.0. Immunoprecipitates were boiled in 2x SDS-PAGE sample buffer and analysed by denaturing polyacrylamide gel electrophoresis.

EXAMPLE 7

Biosensor Experiments

Binding studies and epitope mapping were performed on the optical biosensor (BIAcore™, Pharmacia, Uppsala) using proteins immobilised to a CM5 sensor chip. Immobilisation of *tie*2-EX-FLAG™ and monoclonal antibodies were performed using standard NHS chemistry and using conditions recommended by the manufacturers instructions. Regeneration of the chip surface was carried out by passing 10 μl of 50 mM diethylamine pH 12 over the chip.

EXAMPLE 8

To develop antibodies specifically directed to the extracellular domain of the *tie*2 receptor, a construct was produced containing the *tie*2 extracellular domain fused in frame to the FLAG™ marker peptide. This was achieved by introducing a *BamHI* site at the junction of the extracellular domain and the transmembrane domain of the receptor by site-directed mutagenesis. An oligonucleotide linker sequence encoding the FLAG™ marker peptide, an in frame stop codon and having *BglII* compatible ends was then ligated into this site and successfully ligated plasmids selected by a *ClaI* site within the linker sequence. Expression analysis in COS cells demonstrated a protein of the expected size which could be specifically immunoprecipitated by the M2 (anti-FLAG) gel. The construct was subcloned into the pEE6 CHO cell expression vector for large scale production of the *tie*2-FLAG™ protein. The *tie*2-FLAG™ protein was purified from CHO cell supernatants by affinity chromatography on M2 antibody gel and elution with the free FLAG™ peptide. Subsequent purification by ion-exchange chromatography removed the free peptide and other contaminants giving

a homogenous species of Mr 95,000-100,000 by SDS-PAGE which is consistent with the predicted size of the extracellular domain plus glycosylation. Rats were immunised with the *tie*2-FLAG™ protein and upon achieving an appropriate response their spleen cells were fused with the mouse myeloma NS-1 using standard somatic cell hybridisation techniques. Antibodies were selected on the basis of their reactivity to purified *tie*2-FLAG™ compared to another fusion protein containing the extracellular domain of the NYK receptor ligated to the FLAG™ marker peptide.

To determine if these antibodies could recognise the *tie*2 extracellular domain, cell lines expressing the *tie*2-FLAG™ and *tie*2-AP were biosynthetically labelled with ^{35}[S] Cys-Met and used for immunoprecipitation experiments. Five monoclonal antibodies were shown to specifically immunoprecipitate the *tie*2-FLAG™ and *tie*2-AP protein indicating they recognise the native *tie*2 extracellular domain.

As a further characterization of the antibodies, their ability to detect *tie*2 in Western blotting analysis was studied. All five antibodies were shown to detect the denatured *tie*2 to different degrees. The antibodies have also been shown to react with the *tie*2-FLAG™ coupled to the optical biosensor: epitope mapping experiments have been carried out using the biosensor detection system. With the exception of antibody 4g8, all the antibodies seem to have high affinity for the *tie*2 coupled to the sensor chip. Antibody 3gl bound with such high affinity to the protein that the chip surface could not be regenerated using standard techniques and therefore guanadine-HCl was used to remove bound 3gl antibody.

EXAMPLE 9

Antibody 1ell Fails to Recognise Denatured Material

To characterise the nature of the epitopes recognized by the antibodies in terms of the effect of denaturation of *tie*2 on the binding of antibodies to *tie*2 immobilised on the biosensor chip, the response of the antibodies was determined by passing a solution of antibody across the surface. Figures 4A, 4B and 4C represent response to native *tie*2-FLAG™, while the other three traces (Figure 4D, 4E, 4F) show response following denaturation of the chip with high pH and β-mercaptoethanol. Binding to the denatured *tie*2-FLAG™ is only detected in the case of 4g8 and 3g1, whereas the 1ell antibody exhibits no binding. This result supports

the finding that 1ell exhibits reduced capacity to bind denatured *tie*2-FLAG™ present in the Western blotting analysis (Figure 2).

EXAMPLE 10

Antibody 1ell Causes Dimerization/Oligomerization of *tie*2

The role of the anti-*tie*2 antibodies as an agonist in endothelial cell growth and development would rely on their binding to a growth factor receptor and causing its dimerization and subsequent activation. The *tie*2 receptor is though to be such a receptor, so the action of an antibody which crosslinks *tie*2 may be agonistic. As the physiological effect of ligand stimulation of this receptor is unknown, an artificial system which would detect the dimerization of the extracellular domain was established. A chimeric molecule consisting of the extracellular domain of *tie*2 receptor fused to the intracellular and transmembrane domain of the Erythropoietin receptor (EpoR) was constructed and transfected into a factor dependent cell line (Ba/F3). Previous studies have shown that dimerisation of such a chimeric receptor with the ligand to the RTK can induce signalling via the EpoR cytoplasmic domain and subsequent proliferation of the cells. The inventors' studies have shown that the 1ell antibody was able to stimulate Ba/F3 cells expressing the *tie*2-EpoR chimeric molecule in the absence of IL-3. To quantify this response, an assay was established using dilutions of antibody to stimulate the *tie*2-EpoR expressing Ba/F3 line. A cell line which did not express this chimeric receptor was also included as a control. After four days exposed to the antibody, proliferating cells were quantitated by an assay using MTT (Figure 5). The control cell line is stimulated to proliferate by the antibody - all cells die within 48 hours. The *tie*2-EpoR cell line shows a dose dependent response to the levels of purified 11e1 beginning at a concentration of 0.1 μg/ml. The 4g8 antibody also stimulates the *tie*2-EpoR transfected Ba/F3 cells to a lesser degree, whereas the 3g1 antibody does not stimulate these cells at all (data not shown).

EXAMPLE 11

Antibody 4g8 Can Detect the tie2-2 Receptor on the Surface of Cells by FACS

One powerful role of monoclonal antibodies is their ability to detect the expression of antigens on the surface of cells and study of cells by Fluorescent Activated Cell Sorting (FACS). In order to determine whether the antibodies raised to *tie*2-FLAG™ could be used in this context, the antibodies were tested on cells expressing the *tie*2 receptor. It was found that the 4g8 antibody could detect the extracellular domain of *tie*2 receptor on the surface of COS and Ba/F3 cells. For example, Figure 6 shows the FACS profile of Ba/F3 cells transfected with the *tie*2-EpoR construct. A control cell line gives low fluorescence (Figure 6, unshaded), whereas the transfected cell line shows a higher fluorescence, consistent with the presence of a large number of receptors on the surface (Figure 6, shaded). The 4g8 antibody has also been used detect expression of native *tie*2 on an endothelial cell line of mouse origin, as well as chimeric *tie*2 molecules transfected into COS and Ba/F3 cells, respectively.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: LUDWIG INSTITUTE FOR CANCER RESEARCH

(ii) TITLE OF INVENTION: IMMUNOINTERACTIVE MOLECULES - II

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 805 Third Avenue
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 10022

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch 1.44 Mb storage diskette
(B) COMPUTER: IBM PS/2
(C) OPERATING SYSTEM: PC-DOS
(D) SOFTWARE: Wordperfect

(vi) CURRENT APPLICATION DATA:
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(B) FILING DATE: February 9, 1995
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(C) REFERENCE/DOCKET NUMBER: LUD 5346 PCT

(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: (212) 838-3884

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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1 5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
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(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GATCTGACTA CAAGGACGAC GATGACAAGT GAATCGATA 39

CLAIMS:

1. An immunointeractive molecule capable of binding to otherwise associating with an animal *tie*2/Tek receptor extracellular domain.

2. The immunointeractive molecule of claim 1 wherein said animal is a mammal.

3. The immunointeractive molecule of claim 2 wherein the mammal is a human, livestock animal, laboratory test animal, companion animal or captive wild animal.

4. The immunointeractive molecule of claim 3 wherein the mammal is a human or a murine animal.

5. The immunointeractive molecule of claim 1 wherein the molecule is an antibody.

6. The immunointeractive molecule of claim 5 wherein the antibody is a polyclonal antibody.

7. The immunointeractive molecule of claim 5 wherein the antibody is a monoclonal antibody.

8. The immunointeractive molecule of claim 1 wherein the *tie*2/Tek receptor extracelluar domain is a non-full length molecule fused to a carrier molecule.

9. The immunointeractive molecule of claim 8 wherein the carrier molecule is FLAG™ protein.

10. The immunointeractive molecule of claim 8 wherein the carrier molecule is alkaline phosphatase.

11. A diagnostic agent comprising an immunointeractive molecule capable of binding to or associating with an animal *tie*2/Tek receptor extracellular domain.

12. The diagnostic agent of claim 11 wherein said animal is a mammal.

13. The diagnostic agent of claim 12 wherein said mammal is a human or a murine animal.

14. The diagnostic agent of claim 11 wherein the molecule is an antibody.

15. The diagnostic agent of claim 14 wherein the antibody is a polyclonal antibody.

16. The diagnostic agent of claim 14 wherein the antibody is a monoclonal antibody.

17. The diagnostic agent of claim 14, 15 or 16 further comprising a reporter molecule fused to said antibody.

18. A pharmaceutical composition comprising an immunointeractive molecule capable of binding to or associating with an animal *tie*2/Tek receptor extracellular domain, said composition further comprising one or more pharmaceutical carrier and/or diluent.

19. The pharmaceutical composition of claim 18 wherein said animal is a mammal.

20. The pharmaceutical composition of claim 19 wherein the mammal is a human or a murine animal.

21. The pharmaceutical composition of claim 18 wherein the molecule is an antibody.

22. The pharmaceutical composition of claim 21 wherein the antibody is a polyclonal antibody.

23. The pharmaceutical composition of claim 22 wherein the antibody is a monoclonal antibody.

24. A method for treating an angiogenic-dependent phenotype or disease conditions resulting therefrom in a mammal comprising administering to said mammal an effective amount of an immunointeractive molecule capable of binding to or associating with an animal *tie*2/Tek receptor extracellular domain.

25. The method of claim 24 wherein said mammal is a human.

26. The method of claim 24 or claim 25 wherein the molecule is an antibody.

27. The method of claim 26 wherein said antibody is a polyclonal antibody.

28. The method of claim 26 wherein said antibody is a monoclonal antibody.

29. The method of claim 24 wherein the disease condition is metastasis.

FIG. 1

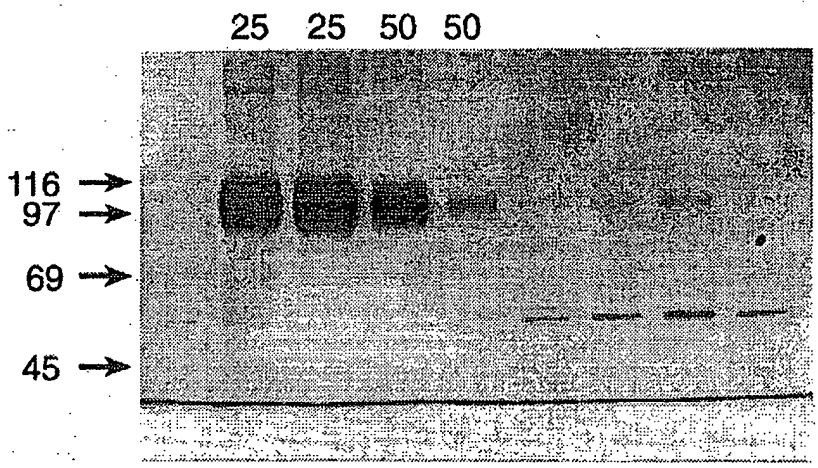

FIG. 2

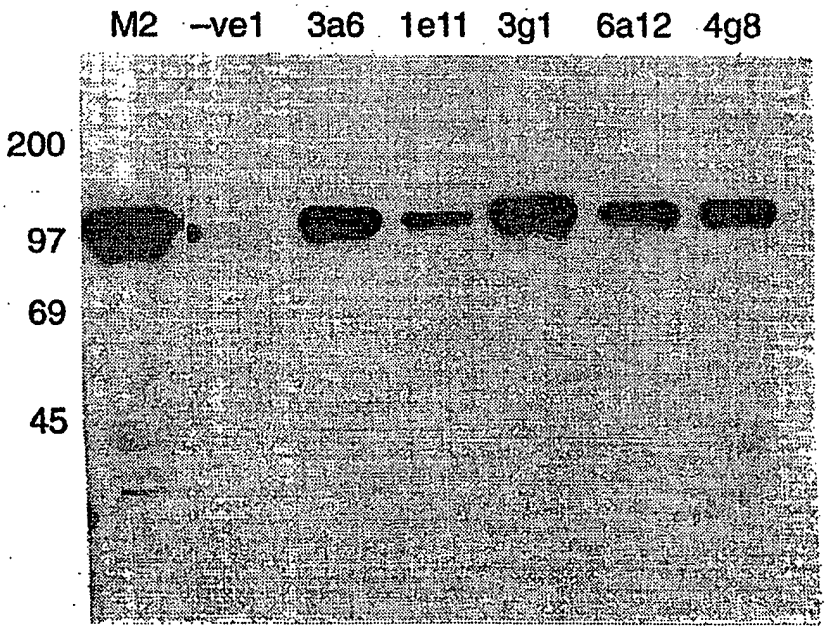

FIG. 3

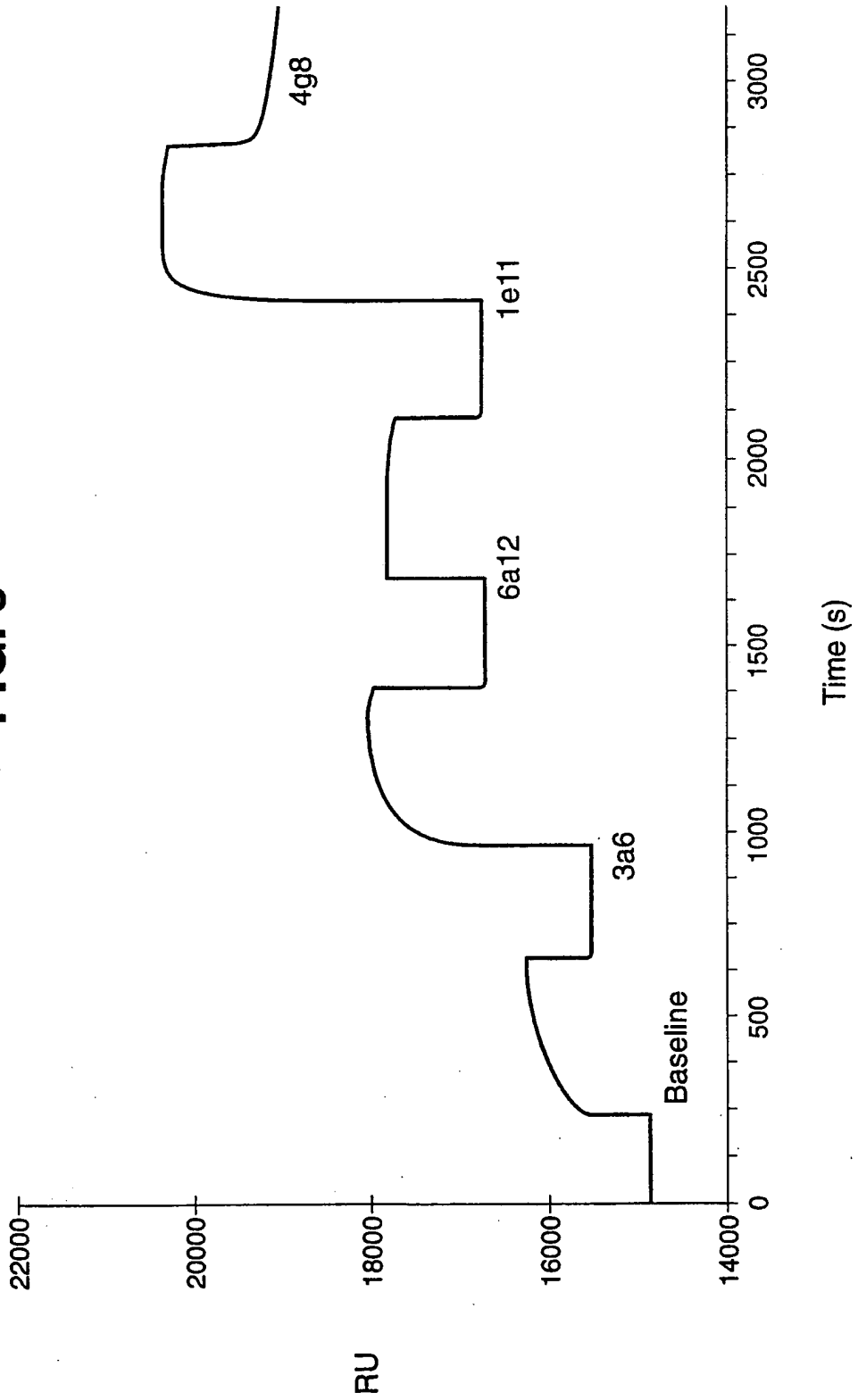
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FIG. 4A

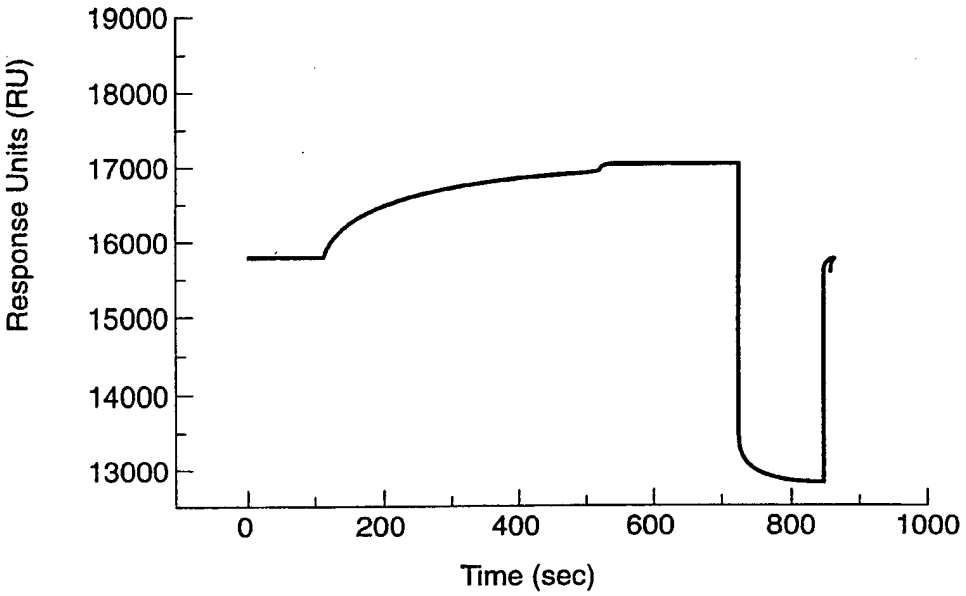
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FIG. 4B

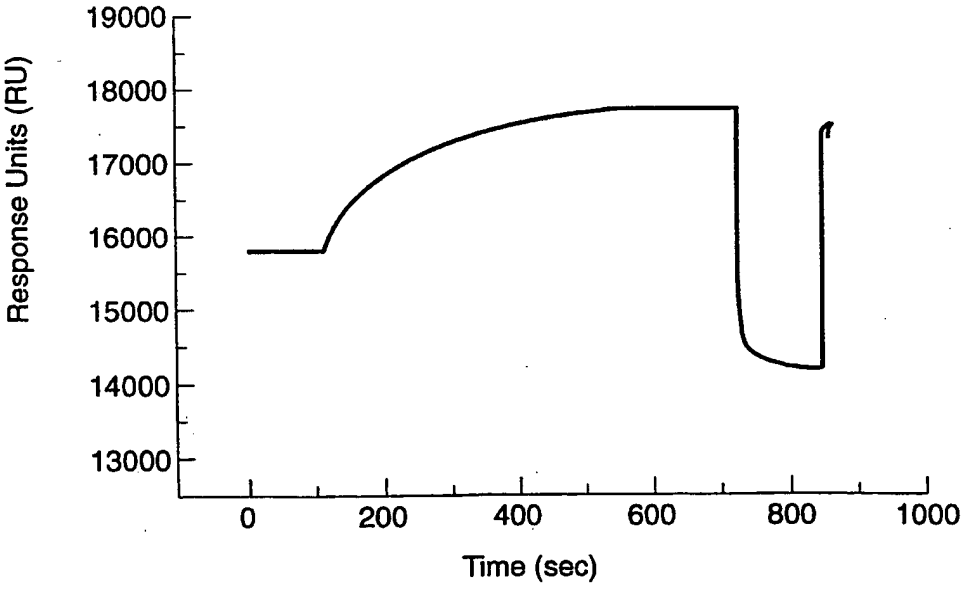
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FIG. 4C

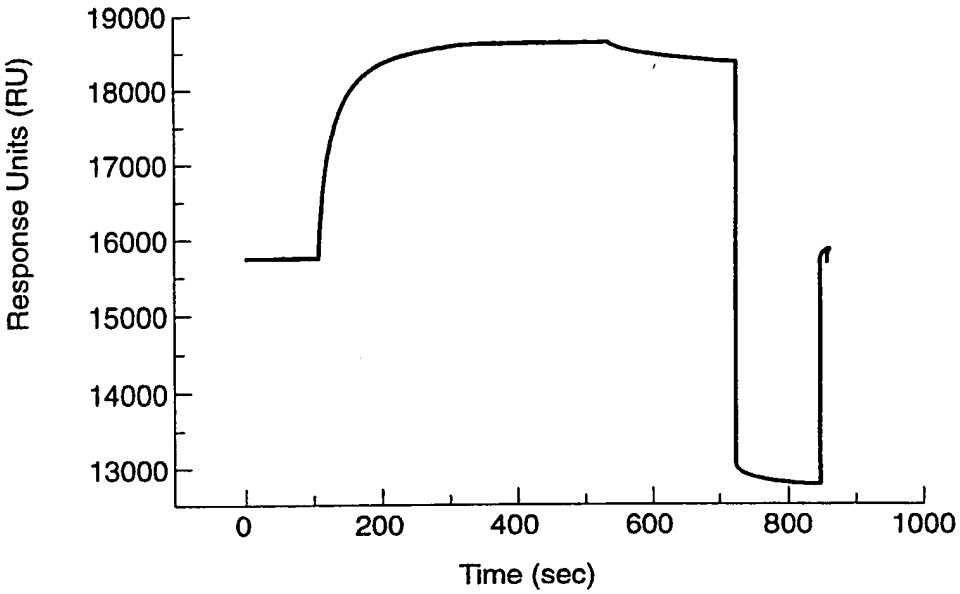
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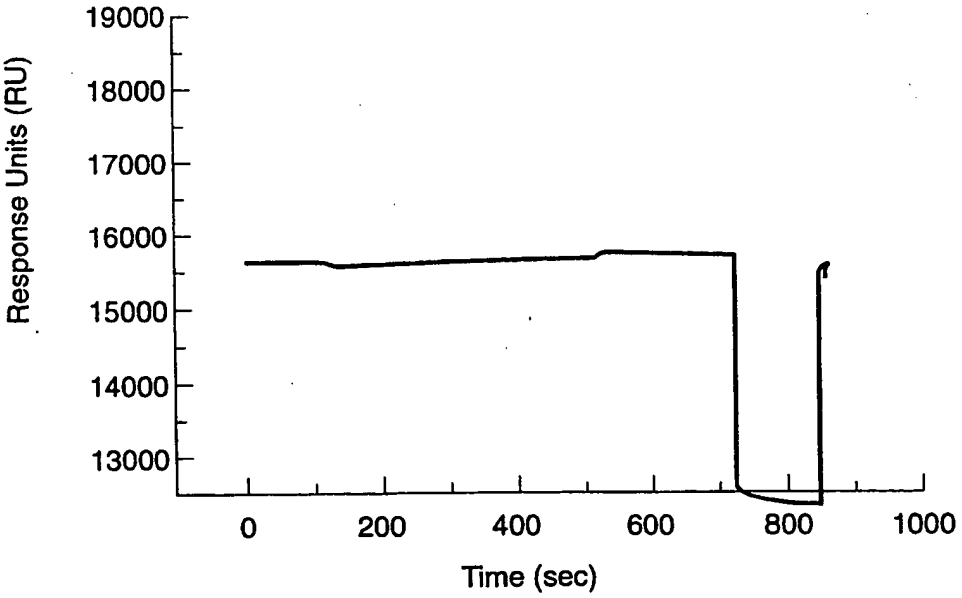
FIG. 4D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13000
0
200
400
600
800
1000
Time (sec)

FIG. 4E

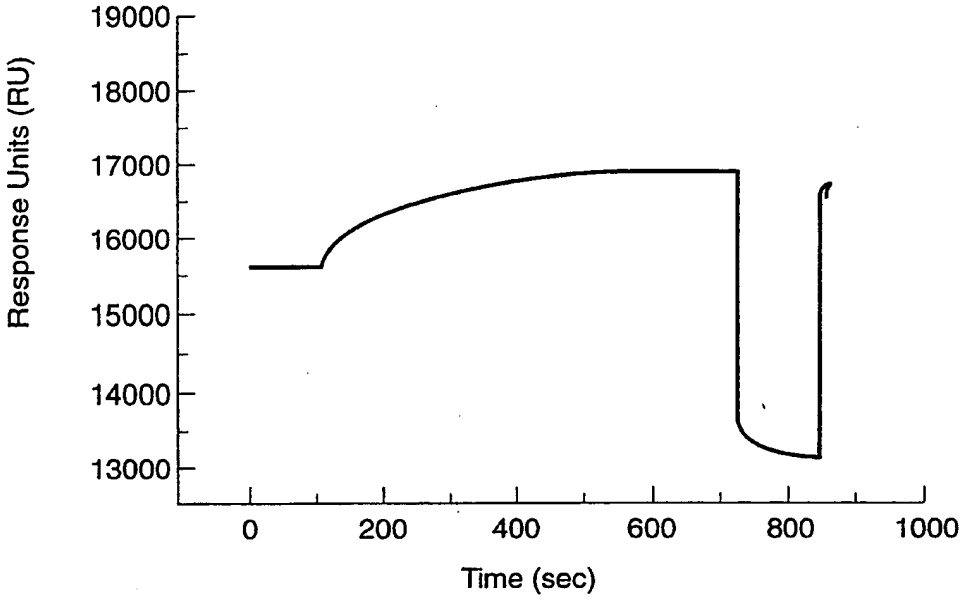

Response Units (RU)
19000
18000
17000
16000
15000
14000
13000
0
200
400
600
800
1000
Time (sec)

FIG. 4F

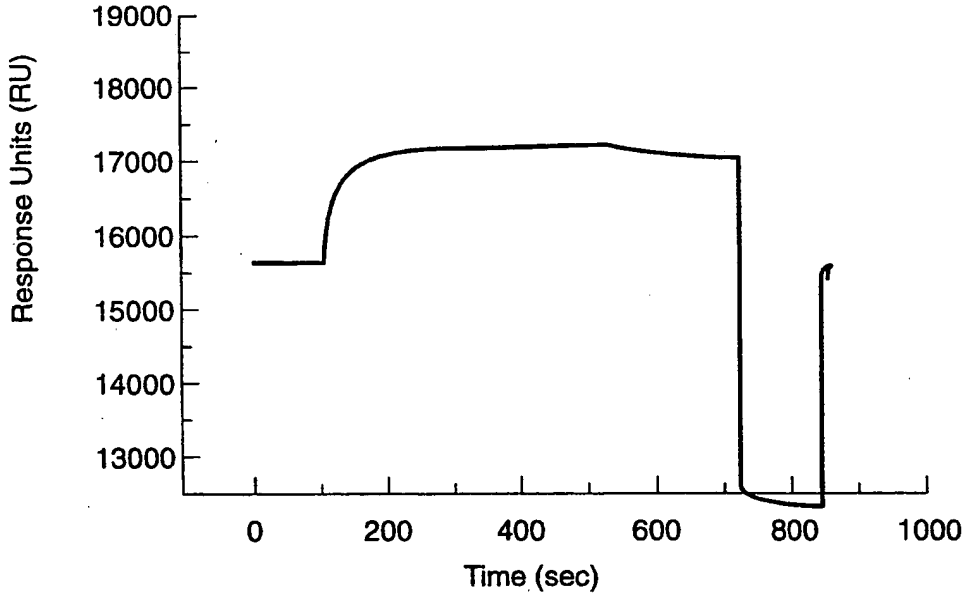

Response Units (RU)
19000
18000
17000
16000
15000
14000
13000
0
200
400
600
800
1000
Time (sec)

FIG. 5

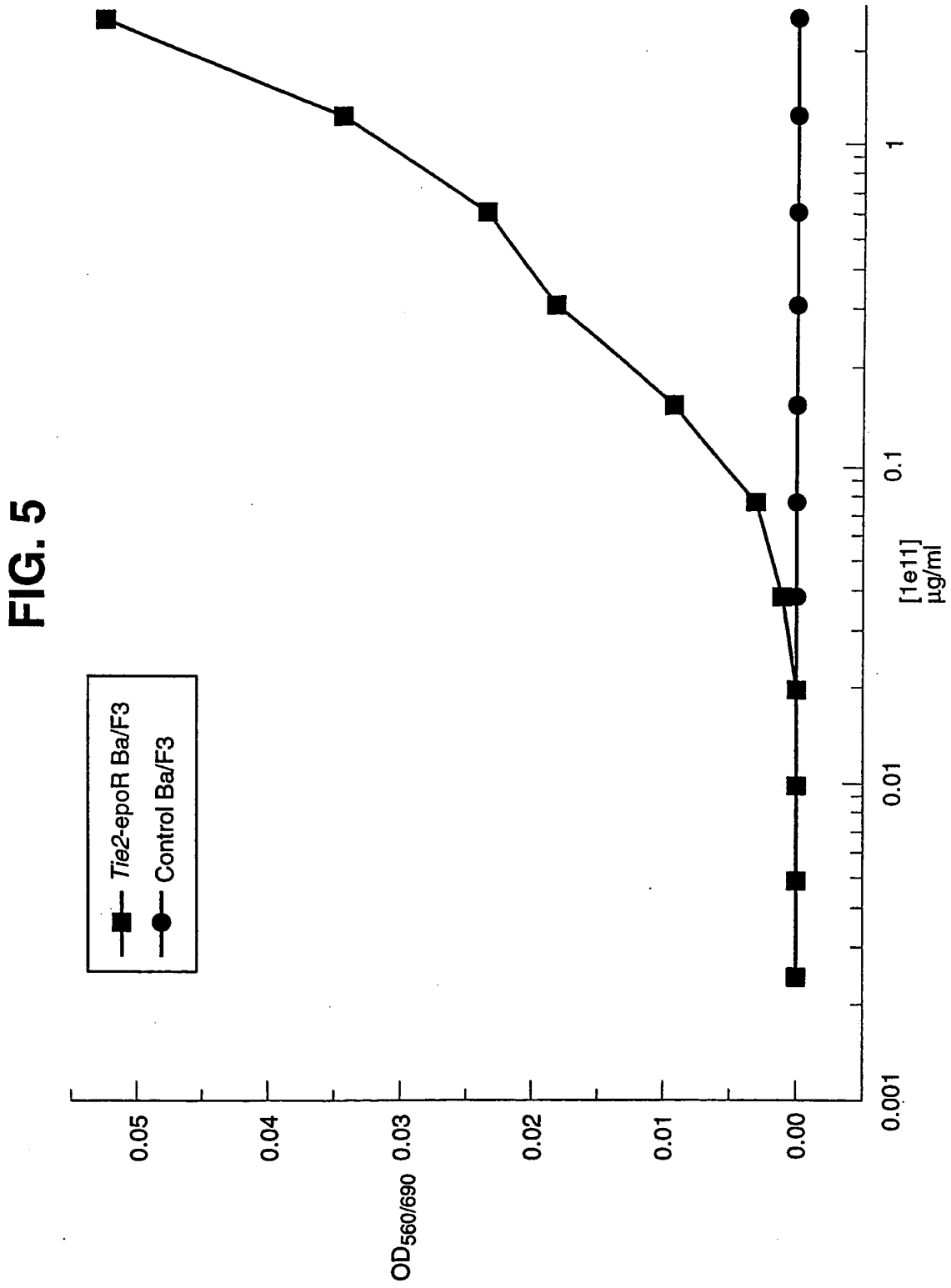
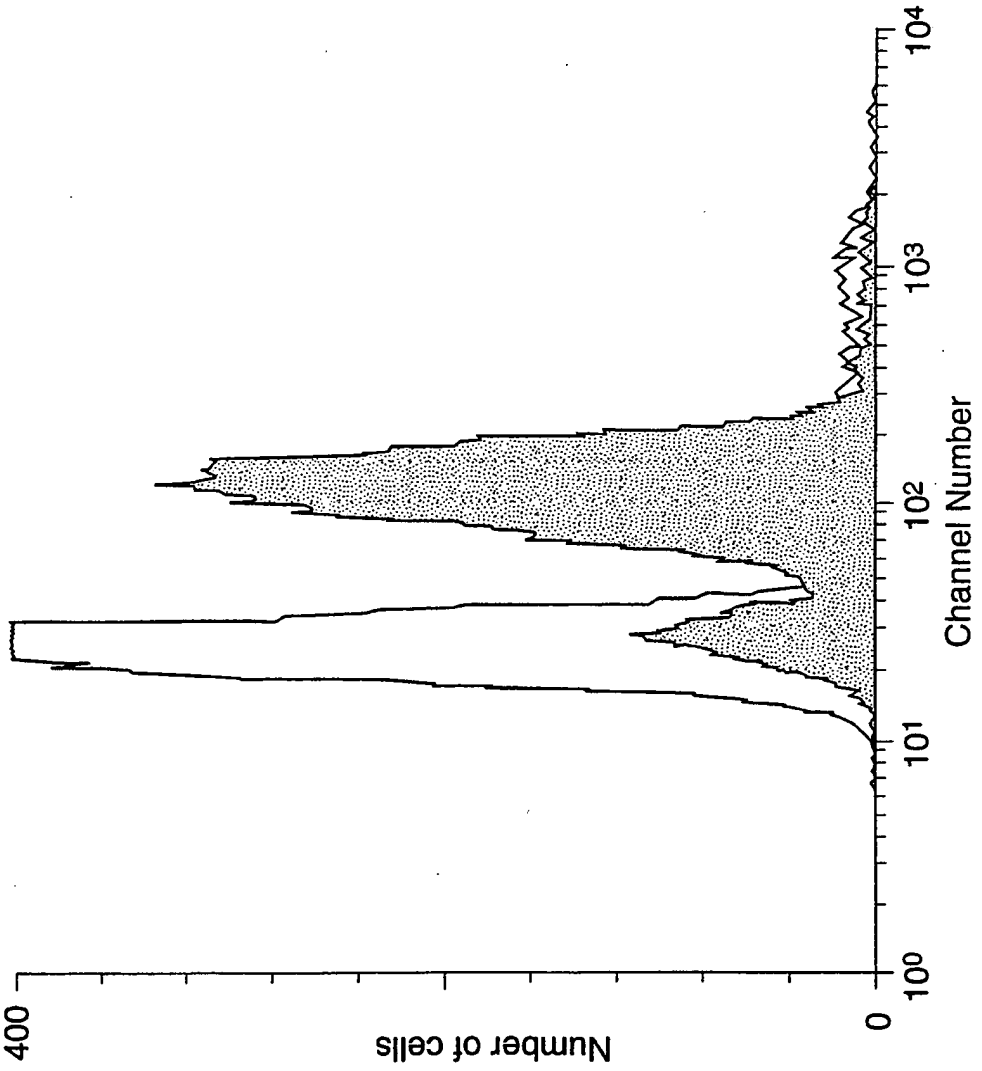

Tie2-epoR Ba/F3
Control Ba/F3
0.05
0.04
0.03
0.02
0.01
0.00
OD560/690
0.001
0.01
0.1
1
[1e11]
µg/ml

FIG. 6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/01743

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :530/388.22, 388.23, 388.24, 810; 424/143.1, 130.1, 158.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/388.22, 388.23, 388.24, 810; 424/143.1, 130.1, 158.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, EMBASE, MEDLINE, CA, BIOSIS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CA, A, 2,085,291 (BREITMAN ET AL) 31 JANUARY 1994, see entire patent.	1-23
Y	US, A, 4,683,295 (CARSON) 28 JULY 1987, see entire document.	1-23
Y	US, A, RE 32,833, (GREENE ET AL) 17 JANUARY 1989, see entire document.	1-29
Y	PROCEEDING NATIONAL ACADEMY OF SCIENCES USA, Volume 90, issued October 1993, Sato et al, "*tie*-1 and *tie*-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system", pages 9355-9358, see entire document.	1-23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
10 MAY 1995	22MAY1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SUSAN A. LORING Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/01743

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DEVELOPMENT, Volume 119, issued 1993, Schnurch et al, "Expression of tie-2, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage", pages 957-968, see entire document.	1-23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/01743

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07K 16/00, 16/18, 16/22, 16/28, 17/00; A61K 39/00, 39/395, 47/00, 49/00; C12Q 1/48; C12N 15/00, 15/54, 9/00, 9/10, 9/12; G01N 33/566